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Induction of Human Papillomavirus-Specific CD4$^+$ and CD8$^+$ Lymphocytes by E7-Pulsed Autologous Dendritic Cells in Patients with Human Papillomavirus Type 16- and 18-Positive Cervical Cancer

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Human papillomavirus (HPV) type 16 (HPV 16) and HPV type 18 (HPV 18) are implicated in the induction and progression of the majority of cervical cancers. Since the E6 and E7 oncoproteins of these viruses are expressed in these lesions, such proteins might be potential tumor-specific targets for immunotherapy. In this report, we demonstrate that recombinant, full-length E7-pulsed autologous dendritic cells (DC) can elicit a specific CD8$^+$ cytotoxic T-lymphocyte (CTL) response against autologous tumor target cells in three patients with HPV 16- or HPV 18-positive cervical cancer. E7-specific CTL populations expressed strong cytolytic activity against autologous tumor cells, did not lyse autologous concanavalin A-treated lymphoblasts or autologous Epstein-Barr virus-transformed lymphoblastoid cell lines (LCL), and showed low levels of cytotoxicity against natural killer cell-sensitive K562 cells. Cytotoxicity against autologous tumor cells could be significantly blocked by anti-HLA class I (W6/32) and anti-CD11a/LFA-1 antibodies. Phenotypically, all CTL populations were CD3$^+$/CD8$^+$, with variable levels of CD56 expression. CTL induced by E7-pulsed DC were also highly cytotoxic against an allogeneic HLA-A2$^+$ HPV 16-positive matched cell line (CaSki). In addition, we show that specific lymphoproliferative responses by autologous CD4$^+$ T cells can also be induced by E7-pulsed autologus DC. E7-specific CD4$^+$ T cells proliferated in response to E7-pulsed LCL but not unpulsed LCL, and this response could be blocked by anti-HLA class II antibody. Finally, with two-color flow cytometric analysis of intracellular cytokine expression at the single-cell level, a marked Th1-like bias (as determined by the frequency of gamma interferon- and interleukin 4-expressing cells) was observable for both CD8$^+$ and CD4$^+$ E7-specific lymphocyte populations. Taken together, these data demonstrate that full-length E7-pulsed DC can induce both E7-specific CD4$^+$ T-cell proliferative responses and strong CD8$^+$ CTL responses capable of lysing autologous naturally HPV-infected cancer cells in patients with cervical cancer. These results may have important implications for the treatment of cervical cancer patients with active or adoptive immunotherapy.

Human papillomavirus (HPV) infection represents the most important risk factor for developing cervical cancer. Although there are over 20 oncogenic HPV genotypes, HPV type 16 (HPV 16) and HPV type 18 (HPV 18) are the more prevalent in cervical cancer regardless of geographical origin (11). The E6 and E7 transforming oncoproteins of these two viruses are detected in the vast majority of HPV-positive cancer biopsies and almost all HPV-containing cell lines and play a crucial role in both transformation and maintenance of the malignant phenotype (for a review, see reference 12). Therefore, E6 and E7 could be ideal candidates as potential tumor-specific targets for cervical cancer immunotherapy.

Several lines of evidence suggest that cell-mediated immune responses are important in controlling both HPV infections and HPV-associated neoplasms. First, there is an increased incidence of associated genital cancer in immunosuppressed patients, while only a minority of genital HPV infections result in the development of cancer in otherwise healthy individuals (32, 39, 41). Second, infiltrating CD4$^+$ (T-helper cells) and CD8$^+$ (cytotoxic or suppressor T cells) T cells have been observed in spontaneously regressing HPV-associated warts. Third, studies with animals have demonstrated that immunized animals are protected from papillomavirus infections and from transplanted tumor cells expressing HPV E6 or E7 oncoproteins (14, 15, 30).

Despite these observations, to date, only a few reports have demonstrated the generation of HPV E6- and E7-specific cytotoxic T lymphocytes (CTLs) in cervical cancer patients, suggesting that CTL precursors may be present at very low levels (1, 19, 36). However, targets naturally infected by HPV (e.g., keratinocytes) are known to express low levels of viral proteins (22, 46) and major histocompatibility complex restriction elements (17, 24) as well as to lack costimulation molecules crucial for naive T-cell priming (35). Therefore, presentation of viral antigens by these nonprofessional antigen-presenting cells (APC) could at least in part explain the capacity of HPV for evading the host immune system (35). Consistent with this hypothesis, hyporesponsiveness or tolerance has been previ-

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ously reported following the presentation of "non-self" antigens by keratinocytes (5, 31, 35).

Studies performed by several groups have recently established the key role played by dendritic cells (DC) in the immune system and have provided a rationale for using DC as natural adjuvants for human immunotherapy (for a review, see reference 45). DC are the most effective APC at activating naive T cells (45, 51), and recently the combination of granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin 4 (IL-4) has been shown to generate large numbers of DC for manipulating the immune response of autologous human T cells (44). In this study, we have used autologous DC pulsed with full-length HPV 16 or HPV 18 E7 oncoprotein to induce an HPV E7-specific T-cell response in HPV 16- or HPV 18-positive cervical cancer patients. Here, using a completely autologous system, we report the in vitro generation of E7-specific proliferative responses by autologous $CD4^+$ lymphocytes as well as the in vitro induction of HLA class I-restricted $CD8^+$ CTLs against naturally HPV 16- or HPV 18-infected autologous cervical tumors in three patients with invasive cervical cancer. In addition, using two-color flow cytometric analysis of intracellular cytokine expression at the single-cell level, we show that a strongly polarized type 1 pattern of cytokine secretion is inducible in the E7-primed $CD8^+$ and $CD4^+$ populations of all three patients.

This report is the first demonstration that class I- and class II-restricted HPV 16 and HPV 18 E7-specific autologous lymphocytes can consistently be induced in cervical cancer patients in vitro by use of full-length E7-pulsed autologous DC. This novel approach overcomes several of the limitations imposed by the use of peptide epitope-based vaccines in an outbred population such as humans and therefore may have important implications for the treatment of cervical cancer patients with active or adoptive immunotherapy.

MATERIALS AND METHODS

Patients. Three patients who had undergone total abdominal hysterectomy for invasive cervical cancer were the sources of tumor tissue and peripheral blood lymphocytes. Specimens were obtained at the time of surgery through the Division of Gynecologic Oncology, Department of Obstretrics and Gynecology, and the Pathology Department at the University of Arkansas for Medical Sciences, Little Rock, under the approval of the Institutional Review Board. HPV typing was performed on fresh tissue biopsy material and on the derived fresh cultures by PCR with sequence-specific primers for HPV 16, HPV 18, HPV type 31, HPV type 33, HPV type 52b, and HPV type 58 (20). Patients 1 and 2 had stage IB2 and IIB squamous cell carcinomas positive for HPV 16 and were 36 and 45 years old, respectively, while patient 3 had stage IB2 adenocarcinoma positive for HPV 18 and was 27 years old. Patients 1 and 2 had received radiation and/or chemotherapy-radiation treatments prior to surgery, while patient 3 had not received any form of therapy prior to surgery.

Tumor cell lines. The natural killer cell (NK)-sensitive target K562 (a human erythroleukemia cell line) and the allogeneic HLA-$A2^+$ HPV 16-positive CaSki cervical cancer cell line (36) were purchased from the American Type Culture Collection, Rockville, Md., and maintained at 37°C in complete medium containing RPMI 1640 (Gibco-BRL, Grand Island, N.Y.) and 10% fetal bovine serum (Gemini Bioproducts, Calabasas, Calif.) in 5% CO_2. Fresh autologous tumor cells were obtained from multiple punch biopsies from all patients. Biopsies were divided into two parts, for histopathologic evaluation and for in vitro studies. Fresh tumor cell lines were maintained in serum-free keratinocyte medium (Gibco-BRL, Grand Island, N.Y.) supplemented with 5 ng of epidermal growth factor per ml and 35 to 50 μg of bovine pituitary extract (Gibco-BRL) per ml at 37°C in 5% CO_2. Briefly, single-cell suspensions were obtained by processing solid tumor samples under sterile conditions at room temperature. Viable tumor tissue was mechanically minced in RPMI 1640 to portions no larger than 1 to 3 mm^3 and washed twice in RPMI 1640. The portions of minced tumor tissue were placed into 250-ml trypsinizing flasks containing 30 ml of enzyme solution (0.14% collagenase type I [Sigma, St. Louis, Mo.] and 0.01% DNase [2,000 kilounits/mg] [Sigma]) in RPMI 1640 and incubated on a magnetic stirring apparatus overnight at 4°C. Enzymatically dissociated tumor was filtered through 150-μm-pore-size nylon mesh to generate a single-cell suspension. The resultant cell suspension was washed twice in RPMI 1640 plus 10% autologous plasma. All experiments were performed with fresh or cryopreserved tumor cultures which had at least 90% viability and contained >99% tumor cells.

HLA phenotypic analysis of $CD8^+$ cultures. HLA class I typing of purified $CD8^+$ cultures was performed by standard lymphocytotoxicity testing (25) in the Tissue Typing Laboratory of the Bone Marrow Transplantation and Blood Transfusion Service at the University of Arkansas for Medical Sciences.

Plasmids and production of E7 proteins. Large amounts of purified E7 oncoproteins of HPV 16 and HPV 18 were generated by use of previously characterized plasmids encoding glutathione *S*-transferase (GST)–E7 fusion proteins (4, 29). Briefly, GST and derivative fusion proteins were maintained in *Escherichia coli* BL21, and protein expression was induced in cultures at an optical density at 600 nm of 0.6 by the addition of isopropyl-β-D-thiogalactoside (IPTG; final concentration, 0.1 mM). Cultures were grown for 6 h for large-scale preparations (2,000 to 4,000 ml). Cells were pelleted, washed once in ice-cold phosphate-buffered saline (PBS; pH 7.4), and resuspended for disruption by sonication on ice in short bursts. Triton X-100 (20%) in PBS was added to a final concentration of 1% and mixed gently for 30 min to aid in the solubilization of the fusion proteins. The bacterial lysate was centrifuged at 12,000 × *g* for 10 min at 4°C, and the supernatant was purified with a glutathione-Sepharose 4B RediPack column in accordance with the procedures suggested by the manufacturer (Pharmacia Biotech, Inc., Piscataway, N.J.). Cleavage of E7 oncoprotein from GST was achieved by use of a site-specific protease (e.g., human thrombin) (Sigma) at 100 U in 2 ml of PBS at room temperature for 16 h. E7 oncoprotein was eluted from the column by spinning (1,000 × *g* for 5 min), and collected material was filter sterilized. The purity of the protein was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Coomassie blue staining, while quantification was obtained spectrophotometrically by the Bio-Rad Laboratories (Hercules, Calif.) protein assay. Preparations were typically >98% pure E7 oncoprotein.

Isolation of PBMC and generation of DC. Peripheral blood mononuclear cells (PBMC) were separated from heparinized venous blood by Ficoll-Hypaque (Sigma) density gradient centrifugation and either cryopreserved in RPMI 1640 plus 10% dimethyl sulfoxide and 30% autologous plasma or immediately used for DC generation. Briefly, PBMC obtained from 42 ml of peripheral blood were placed into six-well culture plates (Costar, Cambridge, Mass.) containing 3 ml of AIM-V (Gibco-BRL) at 0.5×10^7 to 1×10^7 per well. After 2 h at 37°C, nonadherent cells were removed, and adherent cells were cultured at 37°C in a humidified 5% CO_2–95% air incubator with medium supplemented with recombinant human GM-CSF (800 U/ml; Immunex, Seattle, Wash.) and IL-4 (1,000 U/ml; Genzyme, Cambridge, Mass.) (44). In early experiments, only GM-CSF (800 U/ml) was used. Every 2 days, 1 ml of spent medium was replaced by 1.5 ml of fresh medium containing 1,600 U of GM-CSF per ml and 1,000 U of IL-4 per ml to yield final concentrations of 800 and 500 U/ml, respectively (44). After 6 or 7 days of culturing, DC were harvested for pulsing with HPV 16 or HPV 18 E7 oncoproteins as described below.

DC pulsing. Following culturing, DC were washed twice in AIM-V and added to 50-ml polypropylene tubes (Falcon, Oxnard, Calif.). The cationic lipid DOTAP (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) was used to deliver the HPV 16 or HPV 18 E7 proteins into cells. E7 oncoprotein (100 μg/ml) and DOTAP (125 μg in 500 μl of AIM-V) were mixed in polystyrene tubes (12 by 75 mm) at room temperature for 20 min. The complex was added to DC in a total volume of 2 to 5 ml of AIM-V, and the mixture was incubated at 37°C in an incubator with occasional agitation for 3 h. The cells were washed twice in PBS and resuspended in AIM-V as described below.

In vitro generation of HPV E7-specific CTLs. Fresh or cryopreserved responder PBMC were washed and resuspended in AIM-V at 10×10^6 to 20×10^6 cells/well in six-well culture plates with E7-pulsed autologous DC (responder PBMC/DC ratios of 20:1 to 30:1). The cultures were supplemented with recombinant human GM-CSF (500 U/ml) and recombinant human IL-2 (10 U/ml; Aldesleukin; Chiron Therapeutics, Emeryville, Calif.) and incubated at 37°C. Recombinant human IL-2 (10 U/ml) was added to the cultures thereafter every 3 to 4 days. At day 21, $CD8^+$ cells were separated from the bulk cultures by positive selection with CD8-Dynabeads (Dynal Inc., Lake Success, N.Y.) and further expanded in number for 5 to 7 days by use of autologous or allogeneic irradiated PBL (5,000 cGy) (10^6 cells/well) and an anti-CD3 monoclonal antibody (MAb) (Ortho Pharmaceutical Corp., Raritan, N.J.) (0.2 μg/ml) plus 5% autologous plasma and 100 U of IL-2 per ml in 24-well plates (Costar) before being assayed for CTL activity. As negative control targets, autologus lymphoblasts were prepared by 3 days of stimulation with concanavalin A (ConA) (Gibco-BRL; 1 μg/ml) in RPMI 1640 plus IL-2 (25 U/ml) and 5% autologous plasma, while Epstein-Barr virus (EBV)-transformed autologous lymphoblastoid B-cell lines (LCL) were established by coculturing of PBMC with EBV-containing supernatant from the B95.8 cell line in the presence of 1 μg of cyclosporine (Sandoz, Camberley, United Kingdom) per ml and were maintained in AIM-V supplemented with 10% human AB serum (Gemini Bioproducts).

T-cell proliferation assay. $CD4^+$ T cells derived from day-21 CD8-depleted T-cell populations were restimulated once with E7-pulsed DC at a 20:1 ratio and further purified 2 weeks later by positive selection with CD4-Dynabeads (Dynal) to obtain a population more than 99% pure. Specific lymphoproliferative responses against E7 were tested by use of autologous LCL pulsed with full-length E7 in the presence of DOTAP as described above for DC pulsing. Irradiated (7,500 cGy) E7-pulsed or unpulsed autologous LCL were seeded in 96-well plates (2×10^4 cells/well). $CD4^+$ T cells (2×10^4 cells/well) were tested for specific proliferation after 72 h. Cultures were pulsed with 1 μCi of [^{3}H]thymi-

dine per well for the last 16 h, and incorporated radioactivity was measured as described previously (33). HLA restriction of the proliferative responses was investigated by adding an anti-HLA class II MAb (L-243) or an anti-HLA class I MAb (W6/32) (50 μg/ml) (hybridomas were obtained from the American Type Culture Collection). All assays were carried out in triplicate wells.

Cytotoxic activity. A 6-h chromium (^{51}Cr) release assay was performed as previously described (33) to measure the cytotoxic reactivity of E7-stimulated T lymphocytes. In addition to autologous cervical tumor cells, the allogeneic HPV 16-positive CaSki cell line, sharing with patient 1 the HLA-A2 restriction element (36), was used as a target. The K562 tumor cell line was used as a target for the detection of NK activity. Autologous ConA-activated PBL and/or EBV-transformed autologous LCL were used as autologous control targets. To determine the structures on the effector and target cells involved in lysis, MAbs were used to block cytotoxicity. Effector cells were preincubated for 30 min at room temperature with an MAb which recognizes human CD3 (10 μg/ml) or an anti-CD11a/LFA-1 MAb (10 μg/ml) (Pharmingen, San Diego, Calif.) and its IgG1/kappa isotype control MAb against trinitrophenol (10 μg/ml) (Pharmingen). ^{51}Cr-labeled tumor target cells were preincubated with an MAb specific for monomorphic HLA class I (W6/32) (50 μg/ml). The effector cells and ^{51}Cr-labeled target cells were incubated in a final volume of 200 μl of RPMI plus 10% human AB serum/microwell at 37°C with 6% CO_2.

Phenotypic analysis of T cells. Enriched cultures of $CD8^+$ T cells were phenotyped when cytotoxicity was first noted and thereafter in order to correlate cytolytic specificity with a particular lymphoid subset. Flow cytometry was performed with MAbs directly conjugated against the following human leukocyte antigens: Leu-4 (CD3; pan-T cells); Leu-3 (CD4; T helper/inducer); Leu-2a (CD8; T cytotoxic/suppressor); Leu-19 (CD56; NK/K cells); Tac (CD25; IL-2 receptor); HLA-DR (L-243); and T-cell receptor (TCR) α/β or γ/δ (TCR-α/β or TCR-γ/δ, respectively) (Becton Dickinson, San Jose, Calif.). Analysis was done with a FACScan (Becton Dickinson).

Flow cytometric analysis of intracellular cytokines. The protocol for flow cytometry is adapted from that described by Openshaw et al. (37). $CD4^+$ and $CD8^+$ T cells were tested at about 6 weeks after priming, after resting for 14 days after the last antigen stimulation. Briefly, T cells (7.5×10^5/ml) were incubated at 37°C for 6 h in AIM-V plus 5% autologous plasma, 50 ng of phorbol myristate acetate (PMA) per ml, and 500 ng of ionomycin per ml. Brefeldin A (10 μg/ml) was added for the final 3 h of incubation. Controls (nonactivated cultures) were incubated in the presence of Brefeldin A only. The cells were harvested, washed, and fixed with 2% paraformaldehyde in PBS for 20 min at room temperature, after which they were washed and stored overnight in PBS at 4°C. For intracellular staining, the cells were washed and permeabilized by incubation in PBS plus 1% bovine serum albumin and 0.5% saponin (S-7900; Sigma) for 10 min at room temperature. Activated and control cells were stained with fluorescein isothiocyanate (FITC)-labeled anti-gamma interferon (IFN-γ), phycoerythrin (PE)-labeled anti–IL-4, and isotype-matched control (FITC-labeled anti-IgG2a and PE-labeled anti-IgG1) antibodies (Becton Dickinson). After being stained, cells were washed twice with PBS plus 1% bovine serum albumin and 0.5% saponin and once with PBS plus 0.5% BSA and fixed with 2% paraformaldehyde in PBS. Analysis was conducted with a FACScan by use of LYSIS II and WinMDI software (kindly made available by Joe Trotter, Scripps Research Institute, La Jolla, Calif.).

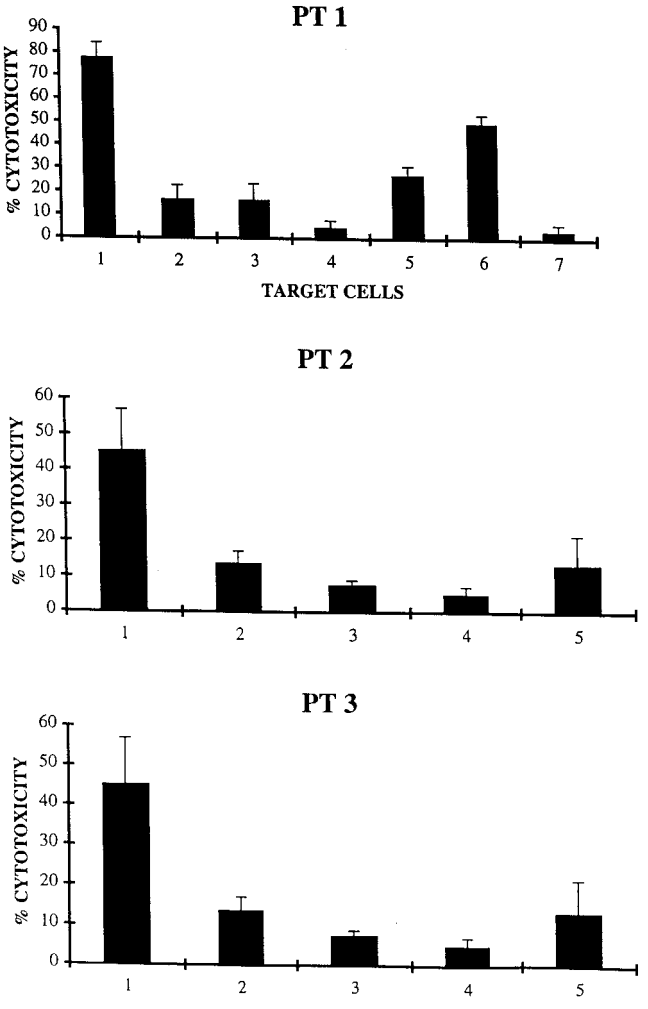


FIG. 1. Tumor-specific HPV 16 and HPV 18 E7-specific $CD8^+$ CTL responses induced by E7-pulsed DC in patients with invasive cervical cancer, as measured in a 6-h ^{51}Cr release assay. Percent lysis (mean ± standard deviation) at a 20:1 effector/target cell ratio is shown. An anti-HLA class I blocking antibody (W6/32) was used at 50 μg/ml, while an anti-CD11a/LFA-1 antibody was used at 10 μg/ml. Bars for patient (PT) 1: 1, autologous tumor; 2, autologous tumor plus W6/32 anti-HLA class I MAb; 3, autologous tumor plus anti-CD11a/LFA-1 antibody; 4, LCL-DOTAP control; 5, LCL-E7; 6, CaSki; and 7, K562. Bars for patients 2 and 3: 1, autologous tumor; 2, autologous tumor plus W6/32 anti-HLA class I MAb; 3, autologous tumor plus anti-CD11a/LFA-1 antibody; 4, LCL control; 5, K562.

RESULTS

HLA typing. PBMC from the cervical cancer patients manifested the following haplotypes: patient 1, HLA-A1, -A2, -B7, -B41, and -CW7; patient 2, HLA-A1, -A2, -B57, -B35, and -CW6; and patient 3, HLA-A1, -A2, -B7, -B41, -CW2, and -CW7.

Tumor-specific $CD8^+$ cytotoxic responses. Cytotoxicity assays were conducted after a minimum of 4 weeks after stimulation of T cells with E7-pulsed DC. For patients 1 and 2, T cells were stimulated with DC pulsed with HPV 16 E7 protein, whereas for patient 3, whose cervical adenocarcinoma carried HPV 18, DC were pulsed with HPV 18 E7 protein. Strong HLA class I-restricted lysis of autologous tumor cells at an effector/target cell ratio of 20:1 was seen for each patient (Fig. 1), while lymphocytes stimulated with DC in the absence of E7 oncoprotein failed to generate specific responses against autologous tumor cells (data not shown). The results presented in Fig. 1 represent the mean of not less than eight assays, with ranges of 67 to 86% lysis for patient 1, 26 to 67% lysis for patient 2, and 36 to 70% lysis for patient 3. Some cytotoxicity against the NK-sensitive cell line K562 was observed, but generally at a low level, particularly so for patient 1. In all cases, minimal cytotoxicity was observed against autologous EBV-transformed LCL (Fig. 1) or autologous ConA-treated lymphoblasts (data not shown). For patient 1, we also show that $CD8^+$ CTLs not only killed autologous tumor cells but also were lytic against E7- and DOTAP-pulsed autologous LCL but not against DOTAP-pulsed LCL controls, confirming the specificity of the cytotoxic response for HPV E7. The lower level of lysis of E7- and DOTAP-pulsed autologous LCL than of tumor cells may be a reflection of the relative inefficiency of the cationic liposome method for antigen introduction into the HLA class I processing and presentation pathway compared with endogenous protein synthesis in tumor cells. We also observed that $CD8^+$ CTLs from patient 1 were cytotoxic against the allogeneic HPV 16-positive CaSki cell line (Fig. 1), which shares HLA-A2 expression with tumor cells from patient 1 (i.e., lysis ranging from 47 to 52% at a 20:1 ratio). This result suggests that the E7-specific $CD8^+$ response in patient 1 is at

least in part HLA-A2 restricted. Blocking studies indicated that in all cases, tumor-specific lysis by $CD8^+$ T cells was inhibited by an MAb specific for HLA class I, the ranges of inhibition being 65 to 91% for patient 1, 67 to 85% for patient 2, and 45 to 82% for patient 3. In addition, we found that an anti-CD11a/LFA-1 MAb but not an anti-CD3 MAb (OKT-3) was also able to block tumor lysis to a significant extent, the ranges of inhibition being 70 to 76% for patient 1, 63 to 65% for patient 2, and 49 to 57% for patient 3. This finding suggests that the CD11a-CD54 adhesion pathway is critical for effective $CD8^+$ T-cell mediated lysis of cervical tumor target cells.

Phenotypic analysis. Flow cytometric analysis was used to determine the phenotypes of the populations of E7-stimulated $CD8^+$ T cells derived from the three patients. All the cells were $CD3^+/CD8^+$ and $CD4^-$, with a variable proportion of CD56 antigen-positive cells. Further analysis revealed the populations to be TCR-α/β^+ (95 to 98%), TCR-γ/δ^+ (2 to 5%), $CD25^+$, $HLA\text{-}DR^+$, and $CD16^-$ (data not shown). The expression of CD56 on T lymphocytes was further analyzed by two-color immunofluorescence (Fig. 2). By this technique, $CD8^+$ T cells were examined for coexpression of CD56. Different percentages of $CD8^+$ T lymphocytes (5 to 55%) coexpressed the CD56 surface antigen during culturing, and the percentage of CD56 expression was shown in repetitive experiments to be strongly correlated with high cytotoxic activity (data not shown). However, CD56 expression on $CD8^+$ T cells did not appear to be a stable phenotype. Indeed, several experiments revealed that expression of this marker was lost by the majority of the previously positive $CD8^+/CD56^+$ T cells when the cells were cultured in low doses of IL-2 but that reexpression took place following restimulation with feeder cells (i.e., autologous or allogeneic irradiated PBL or autologous irradiated tumor cells) (data not shown).

Proliferation assay. E7-stimulated $CD4^+$ T cells (purity, >99%) were tested for specific proliferation against E7-pulsed autologous LCL. As controls, unpulsed autologous LCL or DOTAP-pulsed autologous LCL were used. As shown in Fig. 3, specific proliferation was detectable with E7-pulsed autologous LCL and was significantly higher (P, <0.01) than that induced by LCL alone or DOTAP-pulsed LCL. Finally, E7-specific $CD4^+$ proliferation was significantly inhibited by a MAb to HLA class II molecules (L-243) (Fig. 3) but not by an anti-HLA class I MAb (W6/32) (data not shown), demonstrating the recognition of an epitope(s) presented by an HLA class II molecule(s) of autologous LCL.

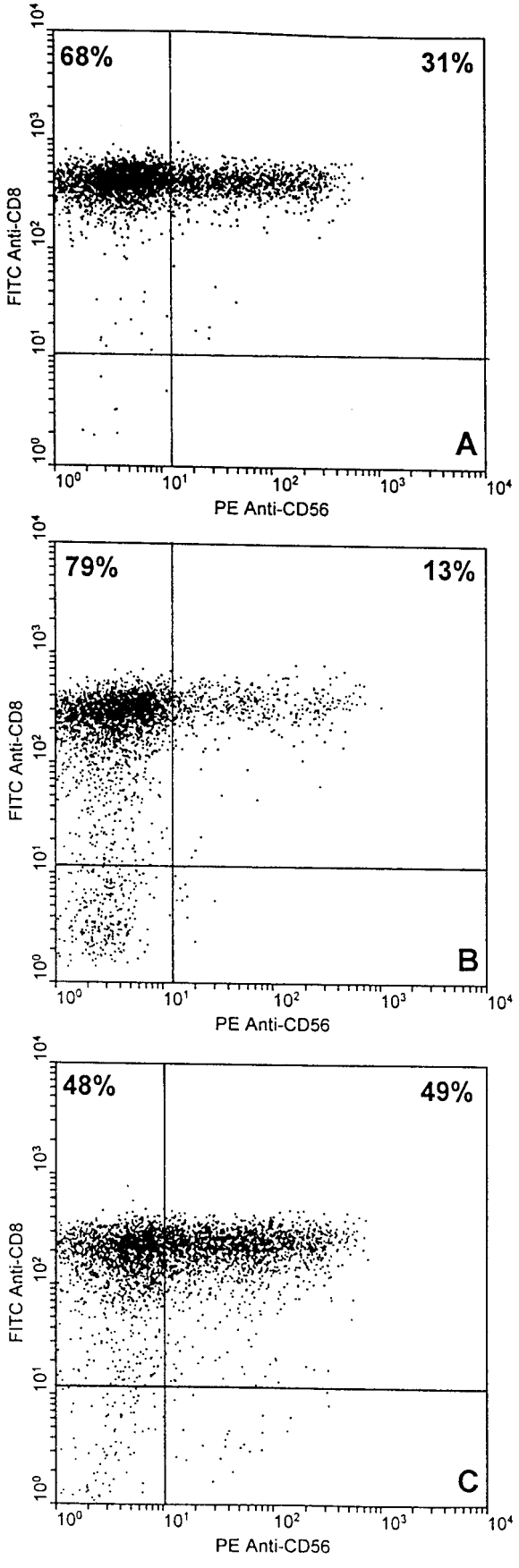
Intracellular cytokine expression by HPV 16 or HPV 18 E7-specific T cells. To evaluate whether cytokine expression from E7-stimulated $CD4^+$ and $CD8^+$ T cells segregated in discrete subsets, we took advantage of recently developed flow cytometric techniques for the detection of intracellular cytokine expression at the single-cell level. Two-color flow cytometric analysis of intracellular IFN-γ and IL-4 expression by CTLs was performed after 6 weeks of culturing and thereafter as described in Materials and Methods. As shown in Fig. 4, the majority of $CD8^+$ T cells contained intracellular IFN-γ but not IL-4, while a second subset contained both intracellular IFN-γ and IL-4 and a third, minor subset contained only IL-4 (Fig. 4). All patients showed similar patterns of cytokine expression in $CD4^+$ T cells (Fig. 5). Similar results were consistently obtained in several repetitive analyses for all patients. Unactivated (i.e., resting) $CD8^+$ or $CD4^+$ T cells from all three patients failed to stain for IFN-γ or IL-4 (Fig. 4 and 5). Similarly, FITC-labeled anti-IgG2a and PE-labeled anti-IgG1 isotype controls did not stain either activated or unactivated $CD4^+$ or $CD8^+$ T cells (data not shown).

DISCUSSION

Until recently, there have been few reports of HPV-specific CTL responses in humans. Most HPV-specific CTLs have been documented in mice, in which HPV is not a natural pathogen (14, 15, 18, 30). This finding has led to the suggestion that HPV has coadapted to the human host by evading the immune system (9). However, HPV-infected epithelial cells could fail to generate CTL responses effectively not because of the lack of specific CTL precursors but because they do not present appropriate levels of antigenic peptides in association with HLA class I molecules and because they fail to express costimulatory molecules necessary for the priming of naive T cells (5, 31, 35). In agreement with this hypothesis, HPV-specific CTLs recognizing HPV oncoproteins have recently been generated from the peripheral blood of cervical cancer patients by a peptide-based epitope approach (1, 10, 19, 42). Unfortunately, cytotoxic responses against naturally HPV-infected autologous tumor cells were not tested in these studies. In this regard, previous reports have warned against the sole use of allogeneic partially HLA-matched cell lines (19, 28) or autologous pulsed or transduced cell lines in vitro (2, 3, 13) as a means to reliably demonstrate specific lysis of targets expressing endogenous antigen. Indeed, CTLs generated by in vitro primary stimulation with high concentrations of peptides often fail to lyse targets expressing endogenous antigen (2, 3, 13, 19).

In this study, we demonstrated that full-length E7-pulsed autologous DC can stimulate a specific $CD8^+$ cytotoxic T-cell response that is capable of killing autologous tumor cells in patients with invasive cervical cancer. We also show that E7-pulsed DC can elicit antigen-specific $CD4^+$ T-cell proliferative responses from the same patients. We chose the E7 oncoprotein of HPV 16 and HPV 18 as a target antigen for the following reasons: (i) HPV 16 and HPV 18 are associated with the vast majority of cervical cancer, and the HPV oncogenic proteins E6 and E7 are important in the induction and maintenance of cellular transformation and are coexpressed in most HPV-containing cervical cancers; and (ii) E7 is a well-characterized cytoplasmic and nuclear protein with little intratypic sequence variation and is more abundant than E6 in HPV-associated cervical cancers. In this study, we took advantage of the cloning and expression in bacteria of E7 as a GST fusion protein. This strategy allowed us to use protein affinity purification under nondenaturing conditions (21) and to produce large amounts of viral antigen for DC pulsing. The use of the cationic lipid DOTAP was shown to be instrumental in the induction of strong and specific $CD8^+$ CTL responses against cervical cancer tumor cells. In this regard, while the intrinsic toxicity of DOTAP for DC was low, its function was presumably to facilitate the cytoplasmic incorporation of exogenous antigen for major histocompatibility complex class I-restricted presentation to $CD8^+$ T cells. Consistent with this view, we found it more difficult to induce specific CTLs against autologous tumor targets when we used DC pulsed overnight with E7 without DOTAP (data not shown).

Currently, epitope-based immunotherapy for cervical cancer has several important limitations. One of the major constraints is that T-cell immune responses to a protein are limited to the epitopes presented by host HLA molecules. This situation imposes severe limitations on the use of synthetic peptides as immunogens in a heterogeneous (i.e., outbred) population such as humans. In contrast, the use of autologous DC pulsed with E7 from the specific HPV serotype involved leaves to the autologous professional APC the processing and presentation of one or more epitopes in association with host HLA molecules.



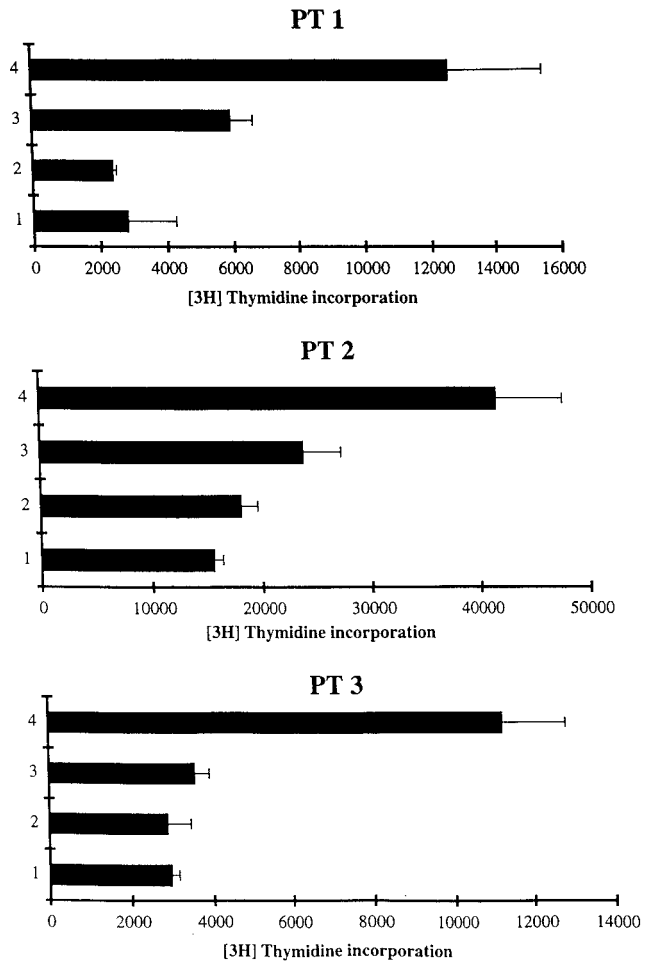


FIG. 3. $CD4^+$ T-cell proliferation in response to stimulation with HPV E7-pulsed autologous irradiated LCL (LCL/E7) in the presence or absence of an HLA class II-specific blocking MAb (L-243, 50 μg/ml). T cells (2×10^4) were cultured in triplicate with LCL/E7 (2×10^4) in a 96-well plate for 72 h. [^{3}H]thymidine (1 μCi/well) was added to each well for at least 16 h of the assay, and proliferation was determined by [^{3}H]thymidine incorporation. Stimulation of $CD4^+$ T cells with irradiated LCL or LCL-DOTAP served as a negative control. Bars: 1, LCL control; 2, LCL-DOTAP control; 3, LCL/E7 plus L243; 4, LCL/E7. The difference in mean proliferation determined by [^{3}H]thymidine incorporation in the presence of LCL/E7 compared to that in the presence of LCL controls is significant at a P value of <0.01, as determined by Student's t test, for all three patients (PT). Data are given as mean ± standard deviation.

The generation of potent CTL immune responses requires the presence of CD4 helper T cells and the presence of both helper and CTL determinants on the same APC (8). Indeed, the inability to mount a potent antitumor immune response has often been attributed to the lack of generation of sufficient tumor-specific T-cell help (6, 38). In recent clinical studies, the in vivo persistence of adoptively transferred antigen-specific $CD8^+$ T cells against cytomegalovirus (50) or the enhanced generation of hepatitis B virus-specific CTLs (49) was dependent upon endogenous CD4 responses. Moreover, the generation of tumor-reactive T-helper cells has been shown to be

FIG. 2. Two-color flow cytometric analysis of CD56 expression by E7-specific $CD8^+$ T cells. T cells were phenotyped when cytotoxicity was first noted as described in Materials and Methods. A representative experiment for each patient is shown. (A) Patient 1. (B) Patient 2. (C) Patient 3.

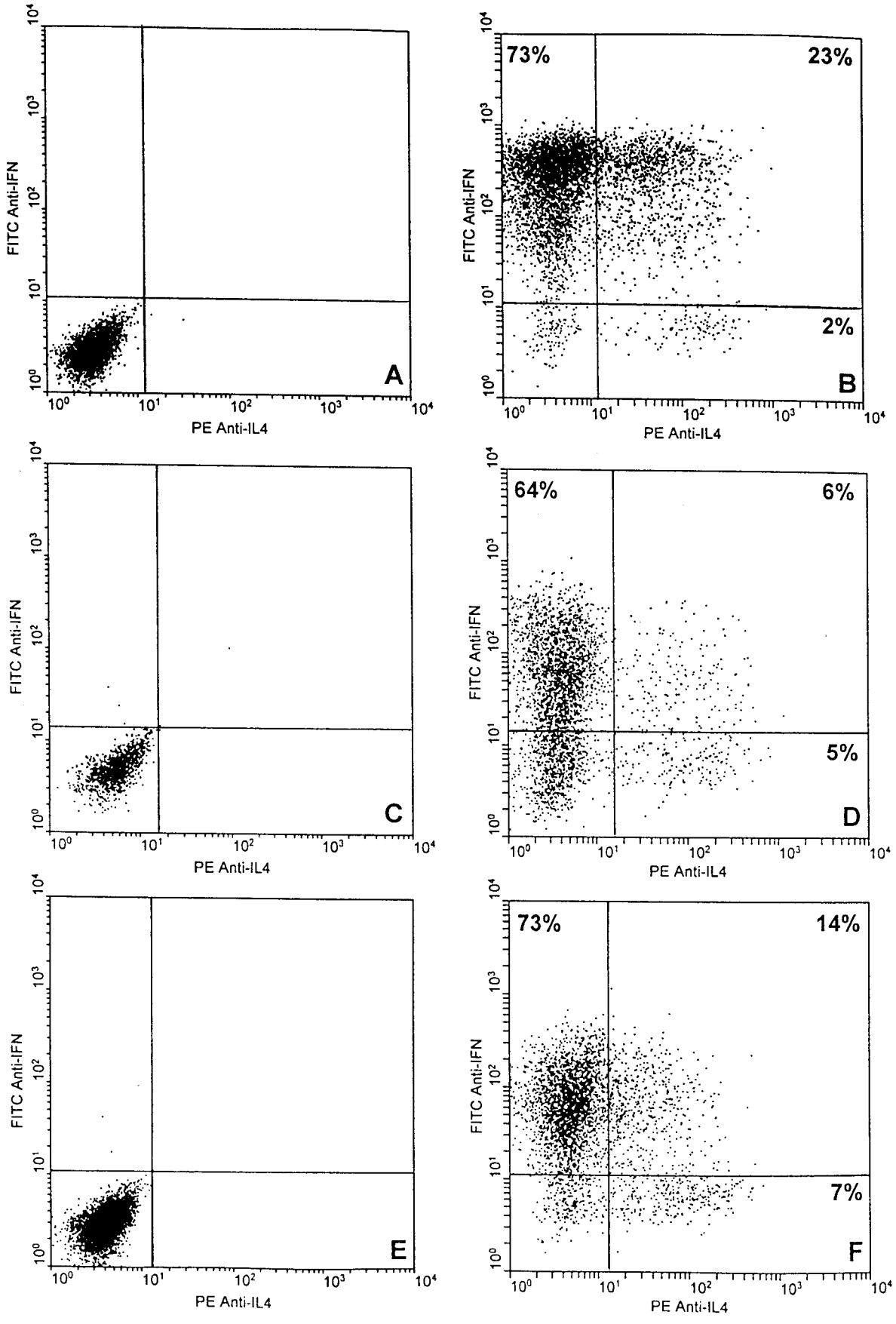


FIG. 4. Two-color flow cytometric analysis of intracellular IFN-γ and IL-4 expression by tumor-specific $CD8^+$ T cells. T cells were tested at about 6 weeks after priming. After resting for 14 days following antigen stimulation, T cells were activated with PMA and ionomycin. T cells were unstimulated (A, C, and E) or stimulated for 6 h with PMA and ionomycin (B, D, and F). (A and B) Patient 1. (C and D) Patient 2. (E and F) Patient 3.

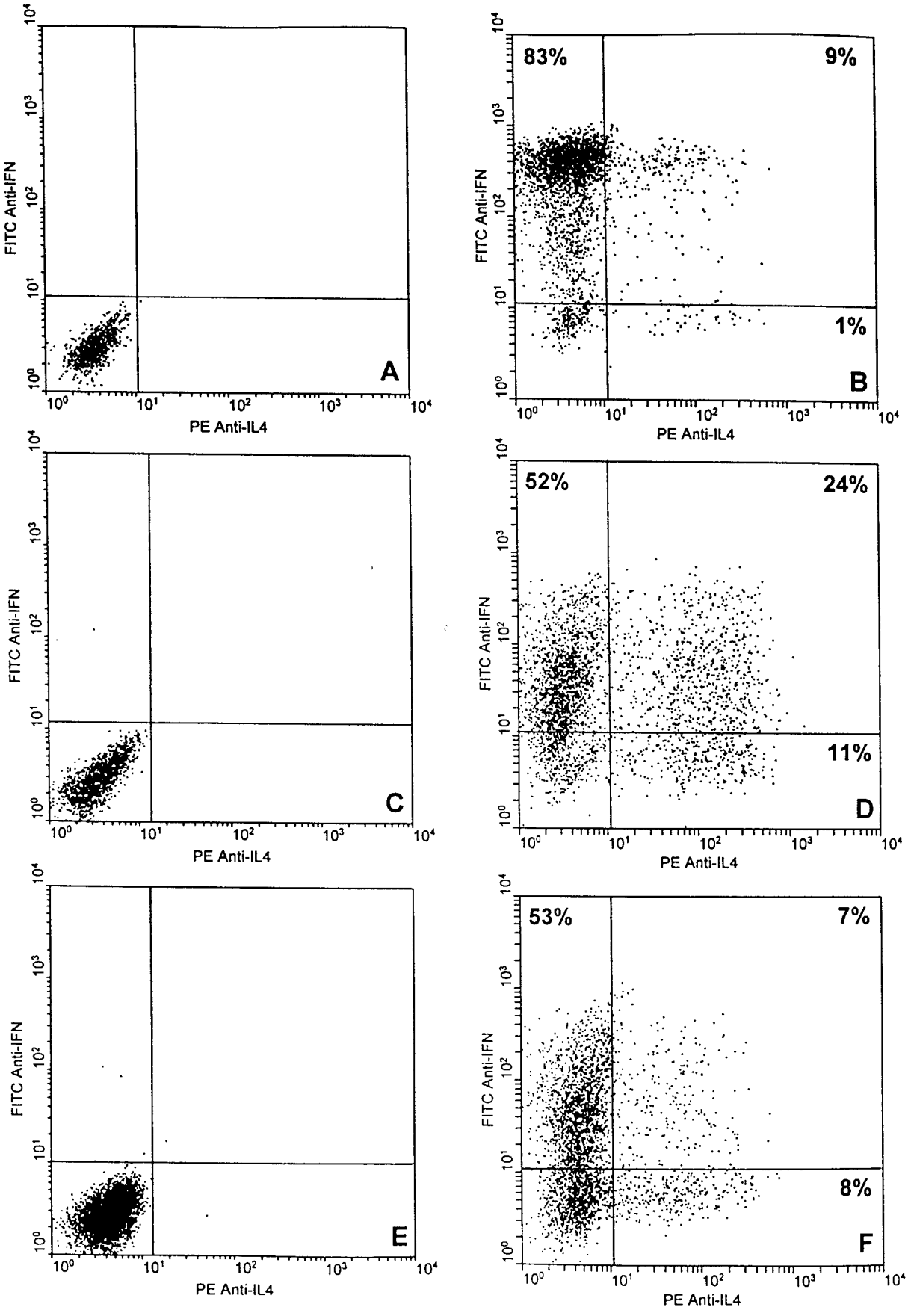


FIG. 5. Two-color flow cytometric analysis of intracellular IFN-γ and IL-4 expression by tumor-specific $CD4^+$ T cells. T cells were tested at about 6 weeks after priming. After resting for 14 days following antigen stimulation, T cells were activated with PMA and ionomycin. T cells were unstimulated (A, C, and E) or stimulated for 6 h with PMA and ionomycin (B, D, and F). (A and B) Patient 1. (C and D) Patient 2. (E and F) Patient 3.

particularly important for the immunotherapy of established (i.e., vascularized) tumors and metastatic disease in several murine tumor models (7, 40). We considered it likely, therefore, that the stimulation of both $CD8^+$ and $CD4^+$ E7-specific T-cell responses would be therapeutically more effective against cervical cancer than that of the $CD8^+$ T-cell response alone. In this study, we show that E7-pulsed DC can readily stimulate E7-specific, HLA class II-restricted proliferative $CD4^+$ T-cell responses, in addition to an effective tumor-specific $CD8^+$ CTL response. Immunotherapy with both $CD4^+$ and $CD8^+$ T cells specific for HPV E7 may thus promote the establishment of long-term tumor-specific immunosurveillance in vivo.

A significant proportion of the autologous tumor-specific cytotoxicity was inhibited by anti-HLA class I antibody. Anti-CD11a/LFA-1 antibody, unlike the anti-CD3 MAb, was also able to significantly block target cell killing by $CD8^+$ CTL. These data therefore indicate that the majority of the cytotoxicity against autologous tumor cells was mediated by antigen-specific HLA class I-restricted CTLs using LFA-1 as an accessory receptor for efficient TCR-dependent target cell recognition (for a review, see reference 47). The level of K562 killing was low or absent for CTLs from patient 1 but was detectable in CTL populations from patients 2 and 3. Autologous LCL were not significantly killed by E7-specific CTLs unless pulsed with E7 oncoprotein, confirming that although these CTLs were highly cytolytic for autologous tumor cells, they failed to kill autologous HPV E7-negative target cells. Finally, when CTLs from patient 1 were tested for their capacity to specifically lyse an allogeneic (but HLA-$A2^+$ HPV 16-positive matched) cervical tumor target cell line (CaSki), strong killing was consistently detectable (range at a ratio of 20:1, 47 to 52%). Lysis of CaSki was significantly inhibited by an anti-HLA class I MAb (W6/32) (range, 58 to 72%), indicating HLA class I-restricted cytotoxicity.

For all three patients, phenotypic analysis of the CTLs revealed a significant $CD8^+/CD56^+$ subpopulation. This observation is in agreement with the results of Hilders et al. (23), who described CD56 expression by $CD8^+$ CTLs derived from tumor-infiltrating lymphocytes from a cervical cancer patient. Although CD56 can be regarded as an NK lineage marker, the CTLs described in this study were HLA class I restricted, unlike the tumor-infiltrating lymphocytes described by Hilders and colleagues (23). Fluorescence-activated cell sorting for $CD56^{high}$ and $CD56^{lo}$ T cells failed to yield stable populations of HLA-restricted or HLA-unrestricted, NK-like $CD8^+$ CTL, although we did observe that $CD56^{high}$ $CD8^+$ T cells were consistently more strongly cytotoxic than $CD56^{lo}$ $CD8^+$ T cells (data not shown). We suggest therefore that CD56 expressed by $CD8^+$ CTL may be an activation antigen associated with cytotoxic function, rather than a lineage-specific marker.

T-cell-mediated protection from viral infection as well as control of tumors is thought to be promoted by type 1 cytokine responses and impaired by type 2 cytokine responses (for a review, see reference 43). In general, type 1 T cells (CD4 or CD8) express IL-2, IFN-γ, and tumor necrosis factor alpha/beta and are cytotoxic, whereas type 2 T cells express IL-4, IL-5, IL-6, IL-10, and IL-13, provide efficient help for B-cell activation, and are noncytotoxic. Consistent with this view, IL-2- and IFN-γ-producing type 1 T cells are believed to promote the development of cell-mediated immunity against HPV-associated neoplasms. Recent studies by Tsukui et al. (48) and Clerici et al. (16) have shown significant dysfunction of type 1 T-cell responses in patients with high-grade cervical intraepithelial lesions and invasive cervical cancer, suggesting that progression to cervical cancer from precursor lesions may be associated with a preferential type 2 T-cell response. In this study, we took advantage of a recently developed flow cytometric technique for detecting intracellular cytokine expression at the single-cell level in HPV 16 or HPV 18 E7-stimulated $CD8^+$ and $CD4^+$ T cells. Two-color flow cytometric analysis of intracellular IFN-γ and IL-4 expression by $CD8^+$ and $CD4^+$ E7-specific T cells demonstrated that HPV-specific T cells from cervical cancer patients showed a major type 1 bias in cytokine expression. Indeed, the majority of cytokine-expressing T cells showed IFN-γ expression, while a minority expressed only IL-4 (Fig. 4 and 5). These findings therefore support the view that even in patients with a progressive HPV infection leading to invasive cervical cancer, the presentation of HPV E7 by DC is still able, at least in vitro, to activate a strong type 1 T-cell response and that type 1 cytokine expression is associated with high cytotoxic activity against autologous tumor cells by $CD8^+$ T cells.

Many recent gene-based strategies for the immunotherapy of cancer have targeted the genetic modification of tumors in order to increase their intrinsic immunogenicity. However, it is becoming increasingly evident that the efficient induction of tumor-specific CTL responses requires the presentation of the relevant antigenic peptides to T cells by professional host APC (27). Recent reports of the use of DC pulsed with specific peptides have already shown great promise for the effective treatment of human malignancies by immunological intervention (26, 34). Taken together, the findings of this study illustrate the feasibility of full-length E7-pulsed DC vaccines or adoptive immunotherapy with E7-specific DC-primed T cells as a powerful strategy for the prevention and treatment of HPV-associated cervical cancer.

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REFERENCES

1. **Alexander, M., M. L. Salgaller, E. Celis, A. Sette, W. A. Barnes, S. A. Rosenberg, and M. A. Steller.** 1996. Generation of tumor-specific cytolytic T lymphocytes from peripheral blood of cervical cancer patients by in vitro stimulation with a synthetic human papillomavirus type 16 E7 epitope. Am. J. Obstet. Gynecol. **175:**1586–1593.
2. **Alexander, M. A., C. A. Damico, K. M. Wieties, T. H. Hansen, and J. M. Connolly.** 1991. Correlation between CD8 dependency and determinant density using peptide-induced, L^d-restricted cytotoxic T lymphocytes. J. Exp. Med. **173:**849–858.
3. **Alexander-Miller, M. A., G. R. Leggatt, and Y. A. Berzofsky.** 1996. Selective expansion of high- or low-avidity cytotoxic T lymphocytes and efficacy for adoptive immunotherapy. Proc. Natl. Acad. Sci. USA **93:**4102–4107.
4. **Antinore, M. J., M. J. Birrer, D. Patel, L. Nader, and D. J. McCance.** 1996. The human papillomavirus type 16 E7 gene product interacts with and trans-activates the AP1 family of transcription factors. EMBO J. **15:**1950–1960.
5. **Bal, V., A. McIndoe, D. Hudson, G. Lombardi, J. Lamb, and R. Lechler.** 1990. Antigen presentation by keratinocytes induces tolerance in human T cells. Eur. J. Immunol. **20:**1893–1897.
6. **Baskar, S., V. Azarenko, E.-G. Marshall, E. Hughes, and S. Ostrand-Rosenberg.** 1994. MHC class II-transfected tumor cells induce long-term tumor-specific immunity in autologous mice. Cell. Immunol. **155:**123–133.
7. **Baskar, S., L. Glimcher, N. Nabavi, R. T. Jones, and S. Ostrand-Rosenberg.** 1998. Major histocompatibility complex class II+ B7-1+ tumor cells are potent vaccines for stimulating tumor rejection in tumor bearing mice. J. Exp. Med. **181:**619–629.
8. **Bennett, S. R. M., F. R. Carbone, F. Karamalis, J. F. A. P. Miller, and W. R.**

Heath. 1997. Induction of a CD8+ cytotoxic T lymphocyte response by cross-priming requires cognate CD4+ T cell help. J. Exp. Med. **186:**65–70.
9. **Beverly, P. C., E. Sadovnikova, X. Zhu, J. Hickling, L. Gao, S. Collins, L. Crawford, K. Vousden, and H. J. Stauss.** 1994. Strategies for studying mouse and human immune responses to human papillomavirus type 16. Ciba Found. Symp. **187:**78–86.
10. **Borysiewicz, L. K., A. Fiander, M. Nimako, S. Man, G. W. G. Wilkinson, D. Westmoreland, A. S. Evans, M. Adams, S. N. Stacey, M. E. G. Boursnell, E. Rutherford, J. K. Hickling, and S. C. Inglis.** 1996. A recombinant vaccinia virus encoding human papillomavirus types 16 and 18, E6 and E7 proteins as immunotherapy for cervical cancer. Lancet **347:**1523–1527.
11. **Bosh, F. X., M. M. Manos, N. Munoz, M. Sherman, A. M. Jansen, J. Peto, M. H. Schiffman, V. Moreno, R. Kurman, and K. V. Shah.** 1995. Prevalence of human papillomavirus in cervical cancer: a worldwide perspective. J. Natl. Cancer Inst. **87:**796–802.
12. **Brinton, L. A.** 1992. Epidemiology of cervical cancer: overview. IARC Sci. Publ. **119:**3–23.
13. **Carbone, F. R., M. W. Moore, J. M. Shell, and M. J. Bevan.** 1998. Induction of cytotoxic T lymphocytes by primary in vitro stimulation with peptides. J. Exp. Med. **167:**1767–1779.
14. **Chen, L., E. K. Thomas, S.-L. Hu, I. Hellstrom, and K. E. Hellstrom.** 1991. Human papilloma virus type 16 nucleoprotein E7 is a tumor rejection antigen. Proc. Natl. Acad. Sci. USA **88:**110–114.
15. **Chen, L., M. T. Mizuno, M. C. Singal, S.-L. Hu, D. A. Galloway, I. Hellstrom, and K. E. Hellstrom.** 1992. Induction of cytotoxic T lymphocytes specific for a syngeneic tumor expressing the E6 oncoprotein of human papilloma virus type 16. J. Immunol. **148:**2617–2621.
16. **Clerici, M., M. Merola, E. Ferrario, D. Trabattoni, M. L. Villa, B. Stefanon, D. J. Venzon, G. M. Shearer, G. De Palo, and E. Clerici.** 1997. Cytokine production patterns in cervical intraepithelial neoplasia: association with human papillomavirus infection. J. Natl. Cancer Inst. **89:**245–250.
17. **Connor, M. E., and P. L. Stern.** 1990. Loss of MHC class-I expression in cervical carcinomas. Int. J. Cancer **46:**1029–1034.
18. **De Bruijn, M. L. H., D. H. Schuurhuis, M. P. M. Vierboom, H. Vermeulen, K. A. J. de Cock, M. E. Ooms, M. E. Ressing, M. Toebes, K. L. M. C. Franken, J.-W. Drijfhout, T. H. M. Ottenhoff, R. Offringa, and C. J. M. Melief.** 1998. Immunization with human papillomavirus type 16 (HPV16) oncoprotein-loaded dendritic cells as well as protein in adjuvant induces MHC class I restricted protection to HPV16-induced tumor cells. Cancer Res. **58:**724–731.
19. **Evans, E. M.-L., S. Man, A. S. Evans, and L. K. Borysiewicz.** 1997. Infiltration of cervical cancer tissue with human papillomavirus-specific cytotoxic T-lymphocytes. Cancer Res. **57:**2943–2950.
20. **Fujinaga, Y., M. Shimada, K. Okazawa, M. Fukushima, I. Kato, and K. Fujinaga.** 1991. Simultaneous detection and typing of genital human papillomavirus DNA using the polymerase chain reaction. J. Gen. Virol. **72:**1039–1044.
21. **Guan, K., and J. E. Dixon.** 1991. Eukaryotic proteins expressed in Escherichia coli: an improved thrombin cleavage and purification procedure of fusion proteins with glutathione-S-transferase. Anal. Biochem. **192:**262–267.
22. **Higgins, G. D., D. M. Uzelin, G. E. Phillips, P. McEvoy, R. Marin, and C. J. Burrel.** 1992. Transcription pattern of human papillomavirus type 16 in genital intraepithelial neoplasia: evidence for promoter usage within the E7 open reading frame during epithelial differentiation. J. Gen. Virol. **73:**2047–2057.
23. **Hilders, C. G. J. M., L. Ras, J. D. H. Van Eendenburg, Y. Nooyen, and G. J. Fleuren.** 1994. Isolation and characterization of tumor-infiltrating lymphocytes from cervical carcinoma. Int. J. Cancer **57:**805–813.
24. **Honma, S., S. Tsukada, S. Honda, M. Nakamura, K. Takakuwa, T. Maruhashi, S. Kodama, K. Konazawa, T. Takahashi, and K. Tanaka.** 1994. Biological-clinical significance of selective loss of HLA-class I allelic product expression in squamous-cell carcinoma of the uterine cervix. Int. J. Cancer **57:**650–655.
25. **Hopkins, K. A.** 1990. Basic microlymphocytotoxicity test, p. 195–201. *In* ASHI laboratory manual. American Society for Histocompatibility and Immunogenetics, Lenexa, Kans.
26. **Hsu, F. J., C. Benike, F. Fagnoni, T. M. Liles, D. Czerwinski, B. Taidi, E. G. Engleman, and R. Levy.** 1996. Vaccination of patients with B-cell lymphoma using autologous antigen-pulsed dendritic cells. Nat. Med. **2:**52–58.
27. **Huang, A. Y. C., P. Golumbek, M. Ahmadzadeh, E. Jaffee, D. Pardoll, and H. Levitsky.** 1994. Role of bone-marrow-derived cells in presenting MHC class I-restricted tumor antigens. Science **264:**961–965.
28. **Jochmus, I., W. Osen, A. Altmann, B. Hofmann, A. Schneider, L. Gissmann, and H.-G. Rammensee.** 1997. Specificity of human cytotoxic T lymphocytes induced by a human papillomavirus type 16 E7-derived peptide. J. Gen. Virol. **78:**1689–1695.
29. **McIntyre, M. C., M. N. Ruesch, and L. A. Laimins.** 1996. Human papillomavirus E7 oncoproteins bind a single form of cyclin E in a complex with cdk2 and p107. Virology **215:**73–82.
30. **Meneguzzi, G., C. Cerni, M. P. Kieny, and R. Lathe.** 1991. Immunization against human papilloma virus type 16 tumor cells with recombinant vaccinia viruses expressing E6 and E7. Virology **181:**62–69.
31. **Molero, I., M. C. Singhal, P. McGowan, H. S. Haugen, J. Blake, K. E. Hellstrom, G. Yang, C. H. Clegg, and L. Chen.** 1997. Immunological ignorance of an E7-encoded cytolytic T-lymphocyte epitope in transgenic mice expressing the E7 and E6 oncogenes of human papillomavirus type 16. J. Virol. **71:**3998–4004.
32. **Nasiell, K., V. Roger, and M. Nasiell.** 1986. Behaviour of mild cervical dysplasia during long term follow-up. Obstet. Gynecol. **67:**665–669.
33. **Nazaruk, R. A., R. Rochford, M. V. Hobbs, and M. J. Cannon.** 1998. Functional diversity of the CD8+ T cell response to Epstein-Barr virus (EBV): implications for the pathogenesis of EBV-associated lymphoproliferative disorders. Blood **91:**3875–3883.
34. **Nestle, F. O., S. Alijagic, M. Gilliet, Y. Sun, S. Grabbe, R. Dummer, G. Burg, and D. Schadendorf.** 1998. Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. Nat. Med. **4:**328–332.
35. **Nikoloff, B. J., and L. A. Turka.** 1994. Immunological functions of nonprofessional antigen-presenting cells: new insights from studies of T-cell interactions with keratinocytes. Immunol. Today **15:**464–469.
36. **Nimaco, M., A. N. Fiander, G. W. G. Wilkinson, L. K. Borysiewicz, and S. Man.** 1997. Human papillomavirus-specific cytotoxic T lymphocytes in patients with cervical intraepithelial neoplasia grade III. Cancer Res. **57:**4855–4861.
37. **Openshaw, P. J. M., E. E. Murphy, N. A. Hosken, V. Maino, K. Davis, K. Murphy, and A. O'Garra.** 1995. Heterogeneity of intracellular cytokine synthesis at the single cell level in polarized T helper 1 and T helper 2 populations. J. Exp. Med. **182:**1357–1364.
38. **Ossendorp, F., E. Mengedé, M. Camps, R. Filius, and C. J. M. Melief.** 1998. Specific T helper cell requirement for optimal induction of cytotoxic T lymphocytes against major histocompatibility complex class II negative tumors. J. Exp. Med. **187:**693–702.
39. **Penn, I.** 1988. Cancers of the anogenital region in renal transplant recipients. Analysis of 65 cases. Cancer **53:**539–545.
40. **Pulaski, B. A., and S. Ostrand-Rosenberg.** 1998. Reduction of established spontaneous mammary carcinoma metastases following immunotherapy with major histocompatibility complex class II and 7.1 cell-based tumor vaccines. Cancer Res. **58:**1486–1493.
41. **Rellihan, M. A., D. P. Dooley, T. W. Burke, M. E. Berkland, and R. N. Longfield.** 1990. Rapidly progressing cervical cancer in a patient with human immunodeficiency virus infection. Gynecol. Oncol. **36:**435–438.
42. **Ressing, M. E., A. Sette, R. M. Brandt, J. Ruppert, P. A. Wentworth, M. Hartman, C. Oseroff, H. M. Grey, C. J. Melief, and W. M. Kast.** 1995. Human CTL epitopes encoded by human papillomavirus type 16 E6 and E7 identified through in vivo and in vitro immunogenicity studies of HLA-A*201-binding peptides. J. Immunol. **154:**5934–5943.
43. **Romagnani, S.** 1992. Human TH1 and TH2 subsets: regulation of differentiation and role in protection and immunopathology. Int. Arch. Allergy Immunol. **98:**279–285.
44. **Romani, N., S. Gruner, D. Brang, E. Kampgen, A. Lenz, B. Trockebacher, G. Konwalinka, P. O. Fritsch, R. M. Steinman, and G. Schuler.** 1994. Proliferating dendritic cell progenitors in human blood. J. Exp. Med. **180:**83–93.
45. **Steinman, R. A.** 1991. The dendritic cell system and its role in immunogenicity. Ann. Rev. Immunol. **9:**271–296.
46. **Stoler, M. H., C. R. Rhodes, A. Whitbeck, S. M. Wolinsky, L. T. Chow, and T. R. Brocker.** 1992. Human papillomavirus type 16 and 18 gene expression in cervical neoplasias. Hum. Pathol. **23:**117–128.
47. **Thiele, D. L., and P. E. Lipsky.** 1989. The role of cell surface recognition structures in the initiation of MHC-unrestricted "promiscuous" killing by T cells. Immunol. Today **10:**375–381.
48. **Tsukui, T., A. Hildesheim, M. H. Schiffman, J. Lucci III, D. Contois, P. Lawler, B. B. Rush, A. T. Loricz, A. Corrigan, R. D. Burk, W. Qu, M. A. Marshall, D. Mann, M. Carrington, M. Clerici, G. M. Shearer, D. P. Carbone, D. R. Scott, R. A. Houghten, and J. A. Berzofsky.** 1996. Interleukin 2 production in vitro by peripheral lymphocytes in response to human papillomavirus-derived peptides: correlation with cervical pathology. Cancer Res. **56:**3967–3974.
49. **Vitiello, A., G. Ishioka, H. M. Grey, R. Rose, P. Farness, R. LaFond, L. Yuan, F. V. Chisari, J. Furze, R. Bartholomeuz, and R. W. Chesnut.** 1995. Development of a lipopeptide-based therapeutic vaccine to treat chronic HBV infection. I. Induction of a primary cytotoxic T lymphocyte response in humans. J. Clin. Investig. **95:**341–349.
50. **Walter, E. A., P. D. Greenberg, M. J. Gilbert, R. J. Finch, K. S. Watanabe, E. D. Thomas, and S. R. Riddell.** 1995. Reconstitution of cellular immunity against cytomegalovirus in recipients of allogeneic bone marrow by transfer of T-cell clones from the donor. N. Engl. J. Med. **333:**1038–1044.
51. **Young, J. W., and K. Inaba.** 1996. Dendritic cells as adjuvants for class I major histocompatibility complex-restricted antitumor immunity. J. Exp. Med. **183:**7–11.